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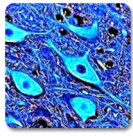
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Kidney ischaemia reperfusion injury in the rat: the EGTI scoring system as a valid and reliable tool for histological assessment

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Abstract

Background: The widely adopted Jablonski scoring system for reporting histological damage in kidney ischaemia reperfusion injury (IRI) provides a limited score documenting the degree of necrosis within tubular cells. IRI is a complex process that involves damage to various cellular components of the renal cortex and therefore there is a need to develop a more detailed scoring system. The aims of this study were to evaluate a new comprehensive histology scoring system (comprising Endothelial, Glomerular, Tubular, and Interstitial (EGTI) components), in rat models of IRI.

Methods: Twenty-seven adult male Lewis rats underwent surgery: Left unilateral IRI (45-minutes left renal pedicle cross-clamping)(n=5); Left unilateral sham(n=5); Bilateral IRI (45-minutes bilateral renal pedicle cross-clamping)(n=9); and Bilateral sham(n=8). Kidney tissue was retrieved at 48 hours. Paraffin sections were examined under H&E staining. RNA extraction and RT-qPCR analysis were performed for acute kidney injury (AKI) markers. Serum creatinine was measured before and at 48 hours post reperfusion for the bilateral model rats.

Results: Forty-five minutes of IRI in the rat caused marked histological damage at 48 hours when compared with sham controls. This was characterized by acute tubular necrosis, endothelial cell disruption and loss, interstitial damage with inflammation, cast formation and necrosis, and glomerular capsule thickening. The EGTI scores correlated significantly with serum creatinine at 48 hours, and expression of AKI markers (NGAL and KIM-1). In addition to this, each individual component on the EGTI histology scoring system correlated significantly with serum creatinine at 48 hours, and expression of NGAL and KIM-1.

Discussion: The EGTI histology scoring system appears reliable and more informative in assessing the degree of damage observed in kidney IRI. Separate scoring parameters for Endothelial, Glomerular, Tubular, and Interstitial injury showed sensitivity to IRI, and permit quantitative analysis of injury and protection in different segments of the kidney.

Keywords: Ischaemia reperfusion injury (IRI), histology scoring system, EGTI scoring system, jablonski score

Introduction

Ischaemia reperfusion injury (IRI) is a complex pathological process characterized by initial restriction of blood supply to the organ followed by subsequent restoration of blood flow and oxygenation, and is an inevitable consequence of organ transplantation, causing significant morbidity and mortality. In kidney transplantation, IRI manifests as primary non-function and delayed graft function, leading to an increased risk of

complications, acute allograft rejection and poorer long-term outcomes [1-3]. Due to the increasing donor shortage problem, expanded criteria donor organs' with the additional risk of increased IRI, are now increasingly used [4].

The pathophysiological processes involved in IRI are complex and include interactions between the endothelium, components of the immune system and cell death programs [3]. Briefly, ischaemia and resultant hypoxia cause an increase

in vascular permeability and impaired endothelial cell barrier function. Cell death programs that consist of cell apoptosis, phagocytosis, and necrosis are activated. Apoptosis causes nuclear fragmentation, loss of mitochondrial integrity and cell shrinkage. Activation of the innate and adaptive components of the immune system, such as complement, contributes significantly to injury [3,5].

In renal IRI, endothelial damage is the result of the initial ischaemia and hypoxia leading to impaired cell barrier function. This causes endothelial swelling and disruption, eventually leading to loss of endothelial integrity [6,7]. Tubular injury is a characteristic feature of kidney IRI since the tubules are most prone to ischaemic damage, and therefore potentially reversible acute tubular necrosis is a hallmark of kidney injury. Specifically, in IRI, hypoxia results in formation of plasma membrane blebs and loss of brush border membrane leading to loss of polarity and integrity of tight junctions. Cast formation occurs secondary to cell death and sloughing. As a result, cast formation and other debris lead to tubular obstruction. These changes, along with tubular necrosis, signify extensive tubular damage [8]. Interstitial damage is also seen in acute kidney injury, but may also result from chronic and progressive damage of varied aetiology. Inflammation, haemorrhage and necrosis are the hallmarks of acute ischaemic damage to the tubulo-interstitium [9]. Thickening of Bowman's capsule, retraction of the glomerular tuft and, in severe cases, glomerular fibrosis are the histological features of ischaemic glomerular damage [10].

Full and comprehensive documentation of these complex features of acute kidney injury is important, to facilitate determination of prognosis following biopsy, and to enable mechanistic studies in this area. The widely adopted 'gold standard' scoring system for reporting histological damage in kidney injury is the Jablonski scoring system [11]. This simple system is designed to give a rapid and objective assessment of kidney injury. In reality, however, it provides a limited score documenting predominantly the degree of necrosis within tubular cells only, ranging from 0 signifying 'no damage', to 4 'necrosis affecting all 3 segments of the proximal convoluted tubule'. It does not provide an assessment of the damage seen within the other cellular components, such as endothelial and glomerular cells. The Jablonski scoring system was originally described following a prolonged recovery period after ischaemic injury, and includes features of recovery (e.g., Tubular cell mitoses) that may not be evident in the typical recovery periods used experimentally, or in clinical samples.

The damage seen in IRI is not limited to tubular cells and includes also endothelial, glomerular and interstitial changes. Exclusive focus on the proximal tubule removes the possibility of detection of varied propensity for injury in these compartments, depending on clinical context and/or experimental therapeutic approach applied. There is a need, therefore, to develop a more detailed scoring system that is simple, reliable and more informative regarding the degree of tissue damage

to the various cellular components of the kidney.

Here, we present the EGTI (Endothelial, Glomerular, Tubular, Interstitial) scoring system, and its analysis in rat models of acute IRI, in comparison with the Jablonski scoring system. In addition, scores were correlated with established acute kidney injury markers neutrophil gelatinase-associated lipocalin (NGAL) and kidney injury molecule 1 (KIM-1), together with serum creatinine as a measure of excretory kidney function, in order to establish their inter-relationships in this model.

Methods

Rat models of IRI

Adult (8 to 12 week old) male Lewis rats weighing 180-220g were used (Harlan Laboratories, UK). The rats were given a 7-day period of acclimatisation to their new environment and were housed and handled according to the local institutional policies and procedures licenced by the Home Office. Analgesia was provided 24 hours pre-op throughout until the time of retrieval in the form of 200mcg of buprenorphine dissolved in 500ml of drinking water. The animals were anaesthetized using Isoflurane and midline laparotomy incisions were made.

Two rat models were used: unilateral IRI and bilateral IRI. In the unilateral model, the left renal pedicle was identified and clamped for 45 minutes using a vascular clip (IRI group). In the bilateral model, both kidneys were identified and their pedicles simultaneously clamped for 45 minutes using vascular clips (IRI group). In both models, the kidneys were visually assessed for both ischaemia upon clamping and subsequent reperfusion upon release of the clamp. Sham controls underwent the same operation without the renal pedicle clamping. During the ischaemic period the abdominal cavity was covered with saline-soaked gauze. The kidneys were retrieved at 48 hours following terminal anaesthesia.

Histology

Kidney tissue was embedded in paraffin and 5 µm tissue sections obtained for Haematoxylin and Eosin (H&E) staining. Histological damage was assessed by an experienced histopathologist blinded as to group allocation (sham versus IRI) and quantified using the EGTI scoring system devised specifically for animal research on kidney tissue in the context of injury (Table 1). The scoring system consists of histological damage in 4 individual components: Endothelial, Glomerular, Tubular, and Interstitial. The scoring was performed in both the preserved and damaged parts of the renal cortex. The Jablonski score was also recorded.

RT-qPCR

Total RNA was extracted using TRIzol reagent (Life technologies) according to manufacturer's instructions. Generation of cDNA was carried out using the High Capacity Reverse Transcription Kit (Life Technologies) with random primers, and RT-qPCR was performed on a ViiA7 Fast Real-Time PCR System (Life Technologies). NGAL and KIM-1 were quantified using

Table 1. The EGTI histology scoring system.

Tissue type	Damage	Score
Tubular	No damage	0
	Loss of Brush Border (BB) in less than 25% of tubular cells. Integrity of basal membrane.	1
	Loss of BB in more than 25% of tubular cells, Thickened basal membrane	2
	(Plus) Inflammation, Cast formation, Necrosis up to 60% of tubular cells	3
	(Plus) Necrosis in more than 60% of tubular cells	4
Endothelial	No damage	0
	Endothelial swelling	1
	Endothelial disruption	2
	Endothelial loss	3
Glomerular	No damage	0
	Thickening of Bowman capsule	1
	Retraction of glomerular tuft	2
	Glomerular fibrosis	3
Tubulo/ Interstitial	No damage	0
	Inflammation, haemorrhage in less than 25% of tissue	1
	(Plus) necrosis in less than 25% of tissue	2
	Necrosis up to 60%	3
	Necrosis more than 60%	4

the POWER SYBR® GREEN PCR Master Mix (Life technologies) with gene-specific primers and normalized to house keeping gene GAPDH. Changes in gene expression were analysed using the 2^{-DDCT} method [12].

Primers' sequences used were:

GAPDH (forward, 5'-CCTCTGACTTCAACAGCGACAC-3'; reverse, 5'-TGTCATACCAGGAAATGAGCTTGA-3'),
NGAL (forward, 5'-GGGCTGTCCGATGAAGTAA-3'; reverse, 5'-CATTGGTCGGTGGGAACAGA-3'),
KIM-1 (forward, 5'-CGGCTAACCAGAGTGACTTGT-3'; reverse, 5'-TACAGAGCCTGGAAGAAGCAG-3'),

Serum creatinine

Serum creatinine in mmol/l was measured using the Jaffe reaction before operation and at time of retrieval in the bilateral IRI model.

Statistical analysis

Statistical analyses were performed using GraphPad Prism v6 software. Data were expressed as mean±SEM or median (and range) and assessed for statistical significance by unpaired Student's *t* test or Mann Whitney U test. The EGTI Histology score and the Jablonski score were correlated with serum creatinine, NGAL and KIM-1. Correlation analysis was reported with significance levels as indicated, where appropriate, in

the figures provided: **p*<0.05, ***p*<0.01, ****p*<0.001.

Results

Histology

Ten rats were used for the unilateral model (5 IRI, 5 sham) and 17 for the bilateral model (9 IRI and 8 sham). Forty-five minutes of unilateral and bilateral IRI in the rat caused marked histological damage at 48 hours when compared with sham controls.

Figures 1A-1C show normal appearance of the renal cortex in a Sham rat. **Figure 1A** shows that the brush border of the tubular cells is intact with no thickening of the basal membrane. No inflammation or necrosis is seen (Tubular score 0). There is no visible interstitium signifying no damage/abnormality within the interstitial compartment (Interstitial score 0). **Figure 1B** shows a uniform endothelium with no swelling or disruption of the endothelial cells (Endothelial score 0). **Figure 1C** shows an intact glomerulus with thin walled Bowman's capsule and no tuft retraction (Glomerular score 0).

Figures 1D-1F show varying degrees of damage of the renal cortex in 45-minute bilateral IRI rats. **Figure 1D** shows inflammation and haemorrhage within the interstitium which is present in less than 25% of the tissue without any evidence of necrosis (Interstitial score 1). **Figure 1E** shows thickened basal membrane of the tubular cells with loss of the brush border in more than 25% of the tubular cells, without the presence of cast formation or necrosis (Tubular score 2). **Figure 1F** shows a glomerulus with evidence of thickened Bowman's capsule (Glomerular score 1), and glomerular tuft retraction (Glomerular score 2).

Figures 1G-1I show varying degrees of damage of the renal cortex in 45-minute unilateral IRI rats. **Figure 1G** shows inflammation and haemorrhage within the interstitial compartment, with necrosis in up to 60% of the cells (Interstitial score 3). **Figure 1H** shows complete necrosis in tubular and interstitial cellular compartments (Tubular score 4, Interstitial score 4). **Figure 1I** shows evidence of inflammation and cast formation within the tubules with evidence of necrosis in up to 60% of tubular cells (Tubular score 3), and endothelial cell disruption and loss (Endothelial score 3).

Figure 2 shows mild damage of the renal cortex in a 45-minute bilateral IRI rat. There is endothelial swelling of the capillaries (A) and the vessel (B) without any disruption (Endothelial score 1).

Unilateral IRI

The median (and range) EGTI histology score was 10 (8-11) in the IRI group compared to 0 (0-1) in the sham group (*p*=0.0079). The mRNA expression of KIM-1 and NGAL was increased 300-fold and 30-fold in the IRI group respectively compared to sham group (*p*<0.0001) [13].

Bilateral IRI

The median (and range) EGTI histology score was 8 (6-9) in the

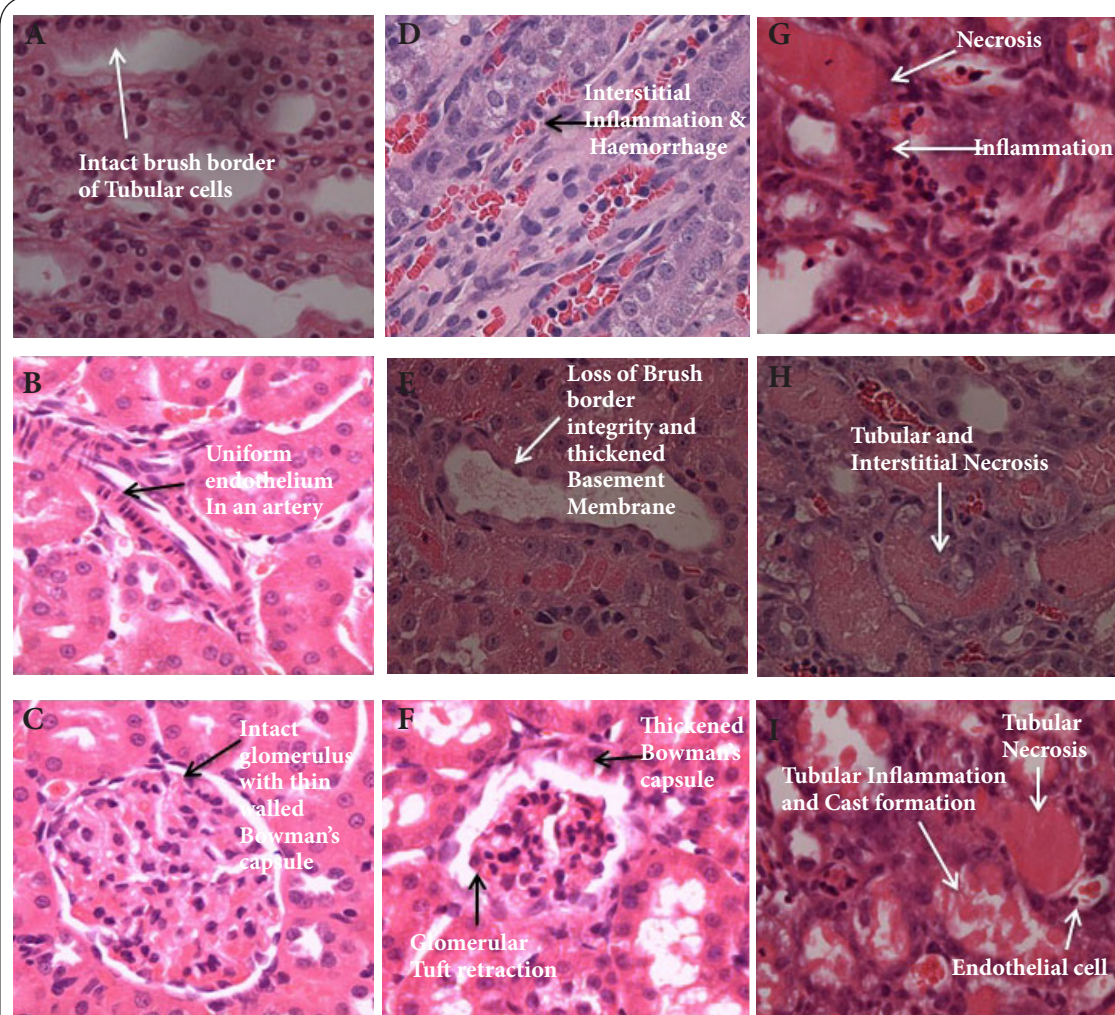


Figure 1. Histological images of rat renal cortex sections.

Renal cortex H&E (x400) paraffin sections, from sham and 45-minute IRI in rats, at 48 hours after reperfusion (Bilateral sham n=8, Bilateral IRI n=9, Unilateral sham n=5, Unilateral IRI n=5) were assessed for Endothelial, Tubular, Glomerular and Interstitial cell damage. (A-C) Normal appearance of renal cortex in a sham rat. (D-F) Mild to moderate damage of the renal cortex in 45-minute bilateral IRI rats. (G-I) Moderate to significant damage of the renal cortex in unilateral 45-minute IRI rats.

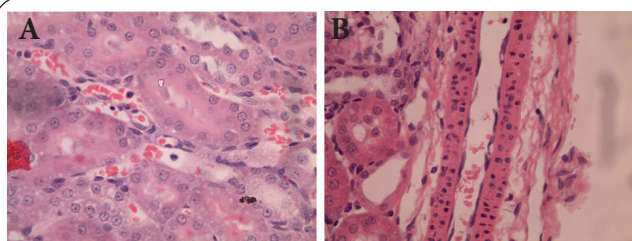


Figure 2. Histological images of endothelial cell swelling in rat renal cortex sections.

Renal cortex H&E (x400) paraffin sections, from sham and 45-minute IRI in rats, at 48 hours after reperfusion (Bilateral sham n=8, Bilateral IRI n=9, Unilateral sham n=5, Unilateral IRI n=5) were assessed for Endothelial, Tubular, Glomerular and Interstitial cell damage. Mild damage of the renal cortex in a 45-minute IRI rat.

IRI group compared to 0 (0-2) in the sham group ($p < 0.0001$). Mean serum creatinine at 48 hours was $76.63(\pm 13.36)$ mmol/l in the IRI group compared with $31.90(\pm 0.25)$ mmol/l in the sham group ($p = 0.0067$). The mRNA expression of KIM-1 and NGAL was increased 150-fold and 14-fold in the IRI group respectively ($p < 0.0001$) (Figure 3). The individual components of the EGTI score were also significantly higher in the IRI group compared with sham (Figure 4).

Correlation of EGTI histology scores and Jablonski scores with serum creatinine, and AKI markers

The EGTI and Jablonski scores both correlated significantly with serum creatinine at 48 hours, and expression of NGAL and KIM-1 (Figure 5). This suggests that both the EGTI score and the Jablonski score are highly predictive of kidney function

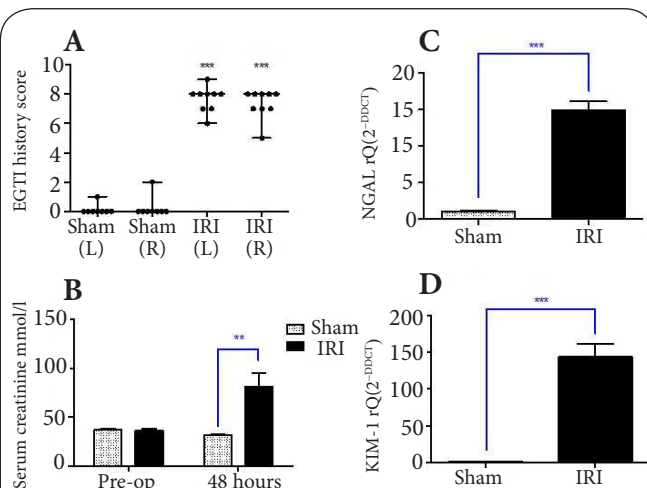


Figure 3. Effect of bilateral IRI on EGTI score, serum creatinine and expression of NGAL and KIM-1. (A) Renal cortex sections from sham and 45-minute bilateral IRI in rats at 48 hours after reperfusion were stained with H&E and assessed using a comprehensive scoring system comprising of Endothelial, Glomerular, Tubular, and Interstitial cell damage (Sham n=8, IRI n=9). Histology scores are plotted as median and range, ***p<0.0001. (B) Serum creatinine was measured pre-op and at 48 hours and is plotted as mean±SEM, **p=0.0077. (C and D) RT-qPCR analysis of KIM-1 and NGAL was performed. Expression is normalized to GAPDH and plotted as mean±SEM, ***p<0.0001.

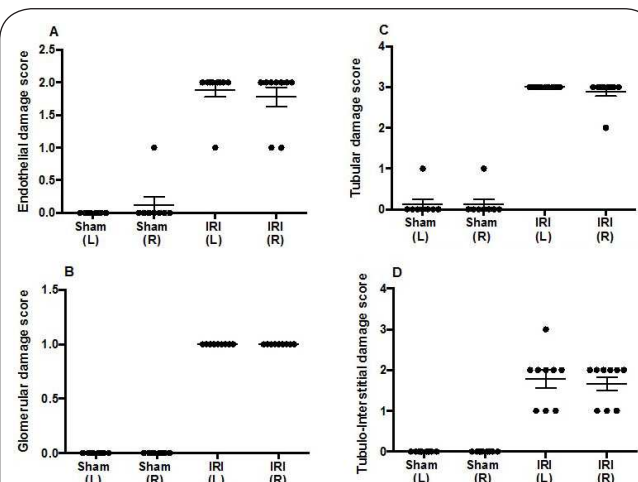


Figure 4. Effect of bilateral IRI on Endothelial, Tubular, Glomerular and Tubulo-Interstitial damages scores. Renal cortex sections from sham and 45-minute bilateral IRI in rats at 48 hours after reperfusion were stained with H&E and assessed using a comprehensive scoring system comprising of Endothelial, Glomerular, Tubular, and Interstitial cell damage (Sham n=8, IRI n=9). Individual cellular component scores are plotted as median and range for Tubular (A), Endothelial (B), Glomerular (C) Tubulo-Interstitial (D) components.

and structural damage.

In addition to this, the association between each component on the EGTI histology scoring system and serum creatinine at 48 hours, and expression of NGAL and KIM-1 was examined using univariate logistic regression analyses. Tubular, endothelial, glomerular, and tubulo-interstitial components were independently associated with serum creatinine at 48 hours (p<0.05), and NGAL and KIM-1 (p<0.0001).

Discussion

This study used rat models of acute IRI to evaluate a new scoring system to document changes in the histological architecture of the renal cortex, established acute kidney injury markers and renal function.

The rat models used in this study are well-established *in vivo* models of acute kidney injury [14,15]. Adult male rats are known to be more susceptible to injury, and so consistent sex selection is an important component [16]. The 45-minutes of cross-clamping of the renal pedicle produces significant but non-fatal, and potentially reversible, injury. The 48-hour reperfusion period is ample to assess the effects of a treatment modality on injury in the acute setting [17], and previous reports have shown that the 45-minute model of IRI used here allows robust and reproducible evaluation of treatment effects on IRI [18,19]. In kidney transplantation most damage occurs in the acute setting, therefore the model used in this study was chosen to reflect this. To ensure that the newly developed scoring system was robust in the presence of functional as well as structural changes as a consequence of IRI, bilateral as well as unilateral IRI models were employed.

The majority of studies investigating kidney IRI report the histological damage according to Jablonski's acute tubular necrosis score [11]. This score is based solely on damage within the tubular cells. It does not document any damage seen to the other cellular components within the renal cortex. Tubular injury is arguably the most characteristic feature of kidney IRI but damage to other structural renal components is also important. The Jablonski scoring system is designed to give a rapid and objective assessment of kidney tubular injury, and is relatively straightforward to use. This system was originally described in a rat model of ischaemia followed by 7 or 14 days of reperfusion [11]. Such a prolonged reperfusion period results in the development of time-dependent morphological features in kidney tissue. For example, a score of '1' includes reference to the presence of mitoses. Mitoses are evident in the presence of tubular cell regeneration, which does not occur in acute models of IRI such as those used in the present study. For acute IRI models, the Jablonski scoring system must therefore be modified to exclude this mitotic criterion. Another significant limitation of the Jablonski system is the absence of brush border observations. One of the first tubular cell changes seen following ischaemic injury is loss of the brush border membrane, prior to cast formation or necrosis.

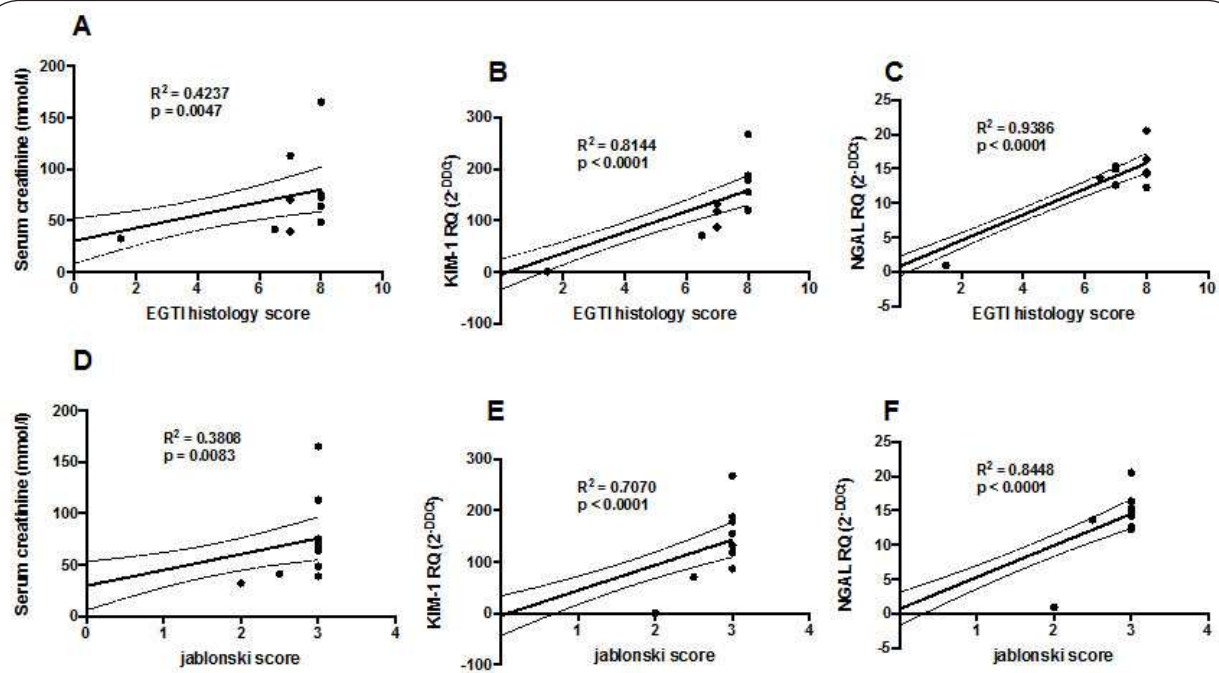


Figure 5. Correlation of EGTI and Jablonski scores with serum creatinine, NGAL and KIM-1. Renal cortex sections from sham and 45-minute bilateral IRI in rats at 48 hours after reperfusion (Sham n=8, IRI n=9) were stained with H&E and scored using a comprehensive scoring system comprising of Endothelial, Glomerular, Tubular, and Interstitial cell damage, in addition to the Jablonski histology score. Serum creatinine was measured at 48 hours and RT-qPCR analysis of KIM-1 and NGAL was performed, with expression normalized to GAPDH. EGTI and Jablonski scores were both plotted and correlated with serum creatinine (A and D), KIM-1 (B and E), and NGAL (C and F).

Kidney IRI is a complex pathophysiological process that causes structural damage to tubular, endothelial, glomerular and tubulo-interstitial cells. Loss of endothelial cell integrity, acute tubular necrosis, tubulo-interstitial damage and glomerular ischaemic damage are all hallmarks of kidney IRI that are important to document fully and comprehensively. It is evident from **Figures 1D-1I** that there is some variation in IRI animals in terms of the damage to various cellular components. This supports further the need for a histology scoring system that reports damage to all of these cellular components.

KIM-1 and NGAL were selected as accurate, robust and reliable biomarkers of acute kidney injury [14,20,21]. It is evident from the histological and acute kidney injury marker changes that the models of IRI used in this study led to significant injury to the kidney. Serum creatinine was selected as a marker of kidney function in the bilateral IRI model. It is the most widely used marker of excretory kidney function, reliably estimating glomerular filtration rate in clinical practice [22]. In this study, changes in serum creatinine between bilateral sham and bilateral IRI rats suggest that this model of acute injury is robust.

The EGTI histology scoring system proposed in this study correlated significantly with established AKI markers and serum creatinine, demonstrating its validity, reliability and sensitivity. Moreover, by reporting changes seen within all of the cellular

components of the renal cortex, our new system offers a greater insight into the severity and range of damage than the Jablonski system. Similarly to the EGTI scores, the Jablonski scores correlated significantly with serum creatinine, NGAL, and KIM-1, although the correlation was slightly less pronounced.

Conclusion

The EGTI histology scoring system described in this paper is a reliable, sensitive and valid tool to assess the degree of damage observed in kidney IRI. It is also more comprehensive than the widely used Jablonski scoring system.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

Authors' contributions	UK	GPC	PN	RJ	TB	DF	RC
Research concept and design	✓	✓	--	--	--	✓	✓
Collection and/or assembly of data	✓	✓	✓	✓	--	--	--
Data analysis and interpretation	✓	✓	✓	✓	✓	✓	✓
Writing the article	✓	--	--	--	--	--	--
Critical revision of the article	✓	✓	✓	✓	✓	✓	✓
Final approval of article	✓	✓	✓	✓	✓	✓	✓
Statistical analysis	✓	--	--	✓	--	--	--

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